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Mapping of RNA$^-$ Temperature-Sensitive Mutants of Sindbis Virus: Assignment of Complementation Groups A, B, and G to Nonstructural Proteins

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Four complementation groups of temperature-sensitive (*ts*) mutants of Sindbis virus that fail to make RNA at the nonpermissive temperature are known, and we have previously shown that group F mutants have defects in nsP4. Here we map representatives of groups A, B, and G. Restriction fragments from a full-length clone of Sindbis virus, Toto1101, were replaced with the corresponding fragments from the various mutants. These hybrid plasmids were transcribed in vitro by SP6 RNA polymerase to produce infectious RNA transcripts, and the virus recovered was tested for temperature sensitivity. After each lesion was mapped to a specific region, cDNA clones of both mutants and revertants were sequenced in order to determine the precise nucleotide change responsible for each mutation. Synthesis of viral RNA and complementation by rescued mutants were also examined in order to study the phenotype of each mutation in a uniform genetic background. The single mutant of group B, *ts*11, had a defect in nsP1 (Ala-348 to Thr). All of the group A and group G mutants examined had lesions in nsP2 (Ala-517 to Thr in *ts*17, Cys-304 to Tyr in *ts*21, and Gly-736 to Ser in *ts*24 for three group A mutants, and Phe-509 to Leu in *ts*18 and Asp-522 to Asn in *ts*7 for two group G mutants). In addition, *ts*7 had a change in nsP3 (Phe-312 to Ser) which also rendered the virus temperature sensitive and RNA$^-$. Thus, changes in any of the four nonstructural proteins can lead to failure to synthesize RNA at a nonpermissive temperature, indicating that all four are involved in RNA synthesis. From the results presented here and from previous results, several of the activities of the nonstructural proteins can be deduced. It appears that nsP1 may be involved in the initiation of minus-strand RNA synthesis. nsP2 appears to be involved in the initiation of 26S RNA synthesis, and in addition it appears to be a protease that cleaves the nonstructural polyprotein precursors. It may also be involved in shutoff of minus-strand RNA synthesis. nsP4 appears to function as the viral polymerase or elongation factor. The functions of nsP3 are as yet unresolved.

Sindbis virus is an alphavirus whose genome is a single-stranded RNA of 11,703 nucleotides of plus polarity (32). During replication, the parental 49S plus-strand RNA is transcribed into a complementary minus-strand RNA which serves as a template for the synthesis of both genome-length RNA and 26S subgenomic mRNA (for a review, see reference 34). The synthesis of both plus- and minus-strand RNA increases during the first 3 h postinfection, but at 3 to 3.5 h after infection the synthesis of minus-strand RNA ceases, whereas the synthesis of both 49S and 26S plus-strand RNAs continues (4, 22). In this early phase, plus-strand RNA is produced in about fivefold molar excess over minus-strand RNA. Replication of viral RNA is presumed to require the activities of four nonstructural proteins, called nsP1, nsP2, nsP3, and nsP4, which are translated from the genomic 49S RNA as two polyprotein precursors that are subsequently processed by posttranslational cleavage (14). It has been postulated that the processing of the nonstructural polypeptides is catalyzed by a virus-encoded protease located within one of the nonstructural polypeptides (for a review, see reference 36).

Temperature-sensitive (*ts*) mutants of Sindbis virus that fail to synthesize RNA at a nonpermissive temperature (called RNA$^-$), some of which are defective in processing of the nonstructural polypeptides, have been isolated and grouped by complementation into four RNA$^-$ groups (A, B, F, and G) (5, 6, 29, 33). These mutants have been presumed to contain *ts* lesions in the viral nonstructural proteins active in viral RNA synthesis. Some of these RNA$^-$ mutants have been characterized in depth (3, 17, 23, 24, 26). Group A mutants were divided by Sawicki and Sawicki (23) into two phenotypic subgroups: subgroup 1 mutants (*ts*15, *ts*17, *ts*21, *ts*24, and *ts*133) were temperature sensitive for synthesis of 26S mRNA, whereas subgroup 2 mutants (*ts*4, *ts*14, *ts*16, *ts*19, and *ts*138) were not. In addition, *ts*17, *ts*24, and *ts*133 failed to cleave the polyprotein precursor nsP123 and failed to shut off minus-strand synthesis upon a shift to 40°C. One member of group G, *ts*18, was also found to be temperature sensitive in synthesis of 26S mRNA and in the cleavage of nsP123, whereas a second mutant of group G, *ts*7, demonstrated neither of these defects. The single mutant in group B, *ts*11, was temperature sensitive for synthesis of minus-strand RNA. Finally, mutant *ts*6 (complementation group F) was found to cease all RNA synthesis upon a shift to a nonpermissive temperature.

We have previously shown that three group F mutants contain lesions in nsP4, suggesting that it is the RNA polymerase or elongation component of the viral replicase (13). Here, we mapped representatives of RNA$^-$ groups A, B, and G by using the same approach. From these results, at least some of the functions of the nonstructural proteins have been deduced.

MATERIALS AND METHODS

Virus growth and purification. The mutants used were obtained originally from B. Burge and had been isolated from the HR strain of Sindbis virus. *ts*17, *ts*21, *ts*24, *ts*11, and *ts*7 were obtained from a stock mutagenized with

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nitrosoguanidine, whereas *ts*18 was isolated after mutagenesis with nitrous acid (5). Viruses were grown in primary or secondary chick embryo cells and harvested 10 to 20 h after infection at 30°C. Revertants of each mutant were isolated by plaquing mutant stocks at 40°C. A single virus plaque was picked from the plate, and the virus was eluted into 1 ml of Eagle medium containing 5% fetal calf serum. This plaque was used to infect primary chicken embryo cells at 40°C and the resulting stocks were plaque assayed at 30 and 40°C and used as the infecting stocks for RNA preparation. Viral RNA was isolated as previously described (21).

Construction of hybrid genomes. cDNA clones of the nonstructural coding regions of mutants and revertants were obtained as previously described (13). Hybrid genomes were produced by replacing restriction fragments in a full-length clone of Sindbis virus, Toto1101 (20), with the corresponding regions from the mutant or revertant cDNA clones (13). Details of the restriction sites used are included in the figure legends. Plasmids containing the large intervals A, B, and C from the mutants, as well as subintervals B1, B2, and B3, were constructed as previously described (13). To obtain recombinant plasmids containing subregions A1 or A2 from the mutants, the restriction fragment *Ssp*I (nucleotide [nt] 504) to *Pst*I (nt 1507) or fragment *Pst*I (nt 1507) to *Cla*I (nt 2713), respectively, of the *ts* mutant was cloned into the shuttle vector πnsP12, which contains the *Sac*I (nt 13552)-to-*Eco*RV (nt 2750) region of Toto1101 cloned into πAN7 (13). The *Sac*I (nt 13552)-to-*Cla*I (nt 2713) fragment of the resulting plasmid was then used to replace the corresponding fragment in Toto1101.

Subintervals B4 and B5 were constructed for *ts*7 to separate two changes found in subinterval B3. Toto:*ts*7B4 contained the *Ava*I (nt 3546)-*Sal*I (nt 4845) fragment of *ts*7 in Toto1101, and Toto:*ts*7B5 contained the *Sal*I (nt 4845)-*Spe*I (nt 5262) fragment of *ts*7 in Toto1101; construction of these two plasmids utilized the shuttle vector Kahn 5B (13).

In vitro transcription and transfection. RNA transcripts were synthesized by transcribing plasmids in vitro with SP6 RNA polymerase (20). The transcribed RNA was assayed for the production of *ts* virus by transfecting confluent monolayers of secondary chicken cells in 35-mm multiwell tissue culture plates (13).

Viral RNA synthesis. RNA synthesis following infection by the various mutants or revertants was assayed at 30°C, at 40°C, or at 40°C after a shift from 30°C by using the cytoplasmic dot hybridization method of White and Bancroft (38).

RESULTS

Construction of recombinant plasmids. In order to localize the *ts* lesions of three group A mutants (*ts*17, *ts*21, and *ts*24), of the sole group B mutant (*ts*11), and of two group G mutants (*ts*7 and *ts*18), we have constructed a number of recombinant plasmids. These recombinant plasmids are derivatives of Toto1101, a full-length cDNA clone of Sindbis virus, from which infectious RNA can be transcribed in vitro with SP6 RNA polymerase (20). Restriction fragments in Toto1101 were replaced with the corresponding fragments from cDNA clones of the mutants, and the resulting constructs are illustrated schematically in Fig. 1; the restriction sites used to construct the hybrid genomes are indicated together with their numbering from the 5′ end of the RNA (32). For gross mapping, the region of the genome encoding the nonstructural proteins was first divided into three large intervals (A, B, and C) as shown. For finer mapping, interval A was subdivided into two subregions, A1 and A2, and region B was subdivided into three overlapping subregions, B1, B2, and B3, as shown. For *ts*7, two additional subregions, B4 and B5, were constructed and tested as described below.

RNA was transcribed from the recombinant plasmids in vitro with SP6 RNA polymerase and transfected onto monolayers of chicken cells. Monolayers were incubated under agarose at 30 or 40°C to determine whether or not the virus recovered was temperature sensitive. The names used to refer to the recombinant plasmids or to the virus derived from them are also indicated in Fig. 1.

Localization of the lesions in group A mutants. The results obtained for the group A recombinant viruses tested are summarized in Table 1. For *ts*17, among the three large-interval replacement clones (Toto:*ts*17A, Toto:*ts*17B, and Toto:*ts*17C), plasmids Toto:*ts*17A and Toto:*ts*17C gave rise to virus that exhibited wild-type growth at the nonpermissive temperature, while *ts* virus was obtained from plasmid Toto:*ts*17B. This localized the *ts* mutation to the interval 2713 to 5262 of the genome. Plasmids Toto:*ts*17B1, Toto:*ts*17B2, and Toto:*ts*17B3 containing three smaller intervals in the B region were then constructed and tested. Plasmid Toto:*ts*17B1 gave rise to temperature-sensitive virus, whereas the other two plasmids did not. Thus, *ts*17 has one or more mutations in the region encoding nsP2, between *Cla*I (nt 2713) and *Ava*I (nt 3546).

By using a similar approach the mutation in *ts*21 was localized to the region between nt 1507 and 2713 of the genome represented in plasmid Toto:*ts*21A2. This region includes the C terminus of nsP1 and the N-terminal half of nsP2.

The lesion in *ts*24 was mapped to the B region. Testing of the subregions then showed that virus from Toto:*ts*24B1 and Toto:*ts*24B2 was temperature sensitive. Assuming that only a single-nucleotide change was involved, the mutation in *ts*24 lies in the overlap between these two subregions, that is, between nt 3546 and 4280 of the genome. Once again, the region included mostly nsP2 sequences, with the N terminus of nsP3 also present.

Localization of the mutation in *ts*11. The results obtained with the *ts*11 constructs tested are summarized in Table 2. By the same procedure as before the *ts*11 lesion was localized to the region between nt 504 and 1507 of the RNA genome, and it is therefore found in nsP1.

Localization of the lesions in group G mutants. The results for *ts*7 and *ts*18 are also shown in Table 2. For *ts*18, the mapping was straightforward and the *ts* lesion was localized to the region between nt 2713 and 3546 of the genome (and was therefore in nsP2).

The results with *ts*7 were more complex, as *ts*7 is a multiple mutant with two lesions contributing to temperature sensitivity. Of the large-interval plasmids tested, only Toto:*ts*7B gave rise to *ts* virus. However, upon testing of the subregions, both plasmids Toto:*ts*7B1 and Toto:*ts*B3 gave rise to *ts* virus, whereas plasmid Toto:*ts*7B2 gave rise to wild-type virus. Thus we conclude that *ts*7 has a *ts* lesion between nt 2713 and 3546 of the genome and a second lesion between nt 4633 and 5262. The first region lies within nsP2, whereas the second lesion lies within nsP3.

Sequence analysis of *ts* mutants and of their revertants. In order to precisely define the *ts* lesions of the six mutants being examined, regions of cDNA clones from each mutant that had been shown by the mapping experiments to contain the *ts* lesions were sequenced by the chemical method (19, 27). In each case, the corresponding region of a revertant

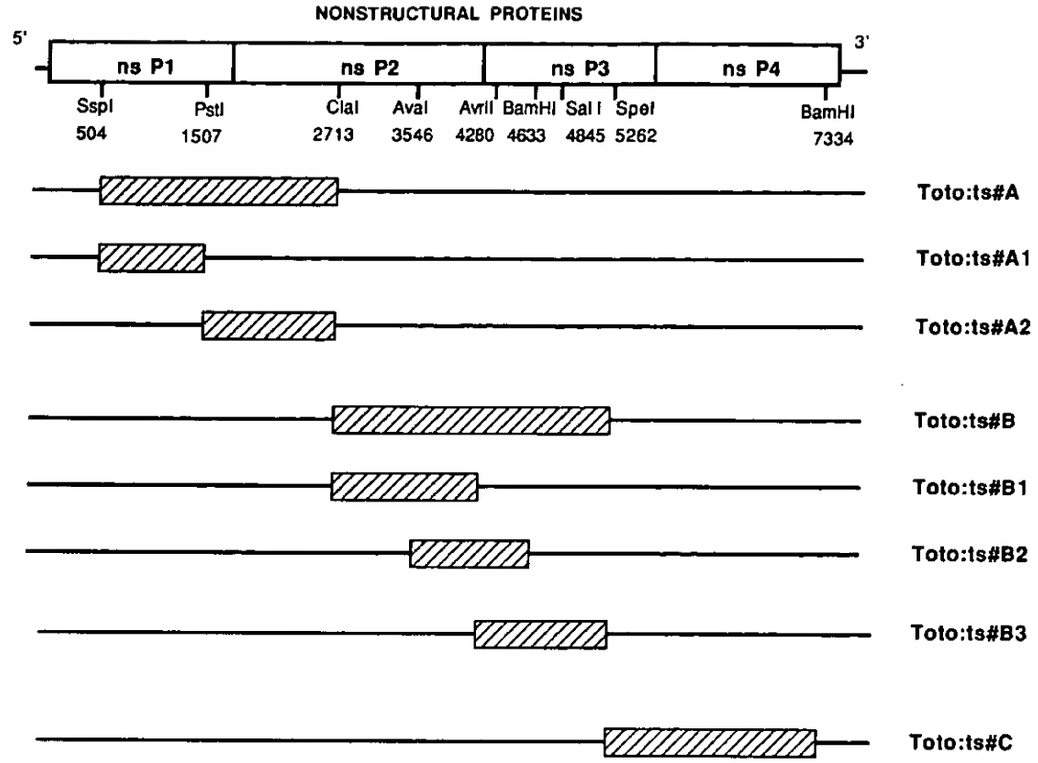

FIG. 1. Construction of hybrid genomes. The top line is a schematic map of the nonstructural protein coding region of Sindbis virus cDNA clone Toto1101. Translated (▭) and nontranslated (——) regions are shown. The boundaries of nonstructural proteins are indicated within the box, as are locations of a number of restriction sites used to construct hybrid genomes (numbered from the 5' end) (32). Below this diagram are shown locations of restriction fragments in clone Toto1101 that were replaced with the corresponding restriction fragments from the *ts* mutants or their revertants (▨). The names used for these clones are shown on the right, e.g., Toto:*ts*11A2 is a construct in which the *Pst*I (nt 1507)-to-*Cla*I (nt 2713) fragment comes from a cDNA clone of mutant *ts*11.

clone was also sequenced. The results obtained are shown in Fig. 2.

Mutant *ts*17 had a single-base substitution in the region sequenced. When we compared the *ts*17 sequence with that of HR and with that of a revertant, we found the mutation responsible for temperature sensitivity to be a change of G to A at nt 3228, which resulted in the replacement of Ala (GCC) at position 517 of nsP2 by Thr (ACC). In the revertant, the

TABLE 1. Summary of group A mutant constructs tested

Recombinant clone	Replaced fragment (nt)	Phenotype[a]
Toto:*ts*17A	504–2713	Wt
Toto:*ts*17B	2713–5262	Ts
Toto:*ts*17B1	2713–4280	Ts
Toto:*ts*17B2	3546–4633	Wt
Toto:*ts*17B3	4280–5262	Wt
Toto:*ts*17C	5262–7334	Wt
Toto:*ts*21A	504–2713	Ts
Toto:*ts*21A1	504–1507	Wt
Toto:*ts*21A2	1507–2713	Ts
Toto:*ts*21B	2713–5262	Wt
Toto:*ts*21C	5262–7334	Wt
Toto:*ts*24A	504–2713	Wt
Toto:*ts*24B	2713–5262	Ts
Toto:*ts*24B1	2713–4280	Ts
Toto:*ts*24B2	3546–4633	Ts
Toto:*ts*24B3	4280–5262	Wt
Toto:*ts*24C	5262–7334	Wt

[a] Wt, Wild type; Ts, temperature sensitive.

TABLE 2. Summary of group B and G mutant constructs tested

Recombinant clone	Replaced fragment (nt)	Phenotype[a]
Toto:*ts*11A	504–2713	Ts
Toto:*ts*11A1	504–1507	Ts
Toto:*ts*11A2	1507–2713	Wt
Toto:*ts*11B	2713–5262	Wt
Toto:*ts*11C	5262–7334	Wt
Toto:*ts*7A	504–2713	Wt
Toto:*ts*7B	2713–5262	Ts
Toto:*ts*7B1	2713–4280	Ts
Toto:*ts*7B2	3546–4633	Wt
Toto:*ts*7B3	4280–5262	Ts
Toto:*ts*7C	5262–7334	Wt
Toto:*ts*18A	504–2713	Wt
Toto:*ts*18B	2713–5262	Ts
Toto:*ts*18B1	2713–4280	Ts
Toto:*ts*18B2	3546–4633	Wt
Toto:*ts*18B3	4280–5262	Wt
Toto:*ts*18C	5262–7334	Wt

[a] Wt, Wild type; Ts, temperature sensitive.

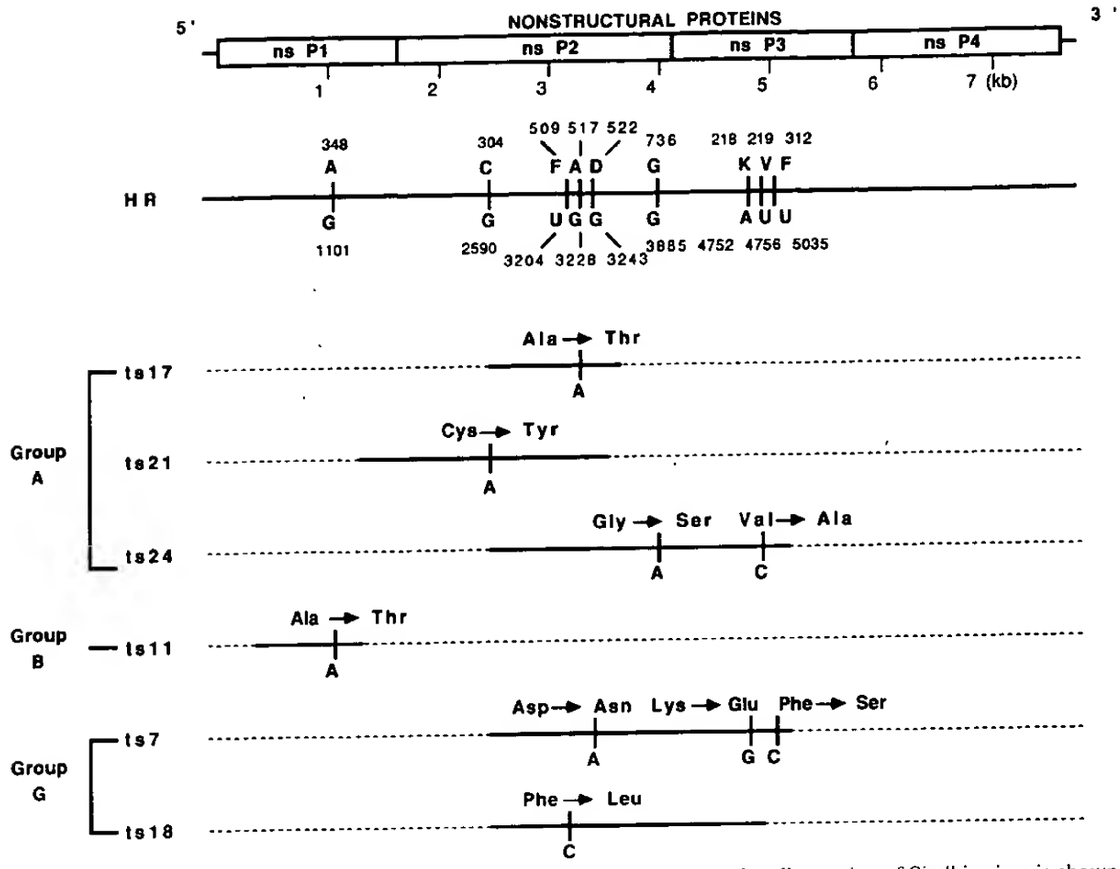

FIG. 2. Localization of the *ts* lesions of six mutants. A schematic of the nonstructural coding region of Sindbis virus is shown. Below are shown sequencing schematics for HR Sindbis virus (32), the parental strain from which the *ts* mutants were isolated (5), and for mutants *ts*17, *ts*21, *ts*24, *ts*11, *ts*7, and *ts*18. Any change from the HR sequence is indicated. Nucleotides (shown below the lines) are numbered from the 5' end of the RNA, and amino acids (shown above the lines) are numbered from the N terminus of each protein (amino acids are indicated in the single-letter code for the HR strain). For each mutant only the region shown by the sold line was sequenced.

altered nucleotide reverted to the parental nucleotide, restoring the parental amino acid. *ts*21 also had a single change in the sequenced region, a G-to-A substitution at nt 2590 of the genome which resulted in the replacement of Cys (UGC) with Tyr (UAC) at position 304 of nsP2. In the *ts*21 revertant that we sequenced, this nucleotide reverted to the original nucleotide.

The *ts* lesion in *ts*24 was found to lie between nt 3546 and 4280 (Table 1). In this region only a single-nucleotide substitution was found, a G-to-A substitution at nt 3885 which resulted in the substitution of Ser (AGC) for Gly (GGC) at position 736 of nsP2. In a revertant of *ts*24 that we sequenced, this nucleotide reverted to the parental nucleotide. In part because of the unusual reversion frequency of *ts*24 (see below), the entire region encompassed by interval B was sequenced to rule out the possibility of a second mutation that might result in temperature sensitivity. During this sequencing we found a second nucleotide substitution, a change of U to C at nt 4756, which resulted in a change of Val (GUC) to Ala (GCC) at position 219 of nsP3 (Fig. 2). This change, which would be present in construct Toto:*ts*24B3, did not lead to temperature sensitivity, however (Table 1). Because the *ts*24 revertant that we sequenced had the parental nucleotide at this position, we suspect that this substitution resulted from cloning a minor variant in the *ts*24 population. In any event, because it did not give rise to temperature sensitivity, it has not been further studied.

The mutation responsible for temperature sensitivity in *ts*11 was found to be a change of G to A at nt 1101, which led to the replacement of Ala (GCC) with Thr (ACC) at position 348 of nsP1. In a revertant that we sequenced, the changed nucleotide had reverted to the original nucleotide, restoring the parental amino acid.

*ts*7 is a double mutant, as described earlier, and the situation with this mutant is complex. Three base substitutions were found in the two regions previously shown to be responsible for temperature sensitivity (Table 2). In the nsP2 region there was a change of G to A at nt 3243, resulting in a change of Asp (GAC) to Asn (AAC) at position 522. In the nsP3 region two changes were found, a change of A to G at nt 4752, resulting in a Lys (AAG)-to-Glu (GAG) substitution at position 218, and a U-to-C change at nt 5035, resulting in a Phe (UUU)-to-Ser (UCU) substitution at amino acid 312. As will be described in more detail below, only the Asp-to-Asn change in nsP2 and the Phe-to-Ser change at position 312 of nsP3 resulted in temperature sensitivity. A partial revertant of *ts*7 was isolated and sequenced and found to retain all three amino acid changes and thus appeared to be a second-site pseudorevertant (see below).

The change in *ts*18 responsible for temperature sensitivity was a U-to-C substitution at nt 3204, resulting in the change of Phe (UUU) to Leu (CUU) at position 509 of nsP2 (Fig. 2). A revertant of *ts*18 was sequenced and found to revert to the original nucleotide, restoring the parental amino acids.

TABLE 3. Efficiency of plaque formation and viral RNA synthesis at 40 and 30°C

Virus	Titer (PFU/ml) at:		EOP[a]	RNA synthesis[b] at:		
	40°C	30°C		40°C	30°C	Shift to 40°C
Toto1101	5.2×10^8	2.0×10^9	2.6×10^{-1}	1.00	1.00	1.00
*ts*17	4.0×10^4	3.0×10^9	1.3×10^{-5}	0.04	1.14	0.19
Toto:*ts*17B1	2.0×10^5	2.0×10^9	8.0×10^{-4}	0.03	1.25	0.27
*ts*21	4.8×10^6	4.9×10^9	1.0×10^{-3}	0.03	0.84	0.32
Toto:*ts*21A2	1.2×10^5	1.2×10^9	1.0×10^{-4}	0.03	0.87	0.40
*ts*24	<10	1.2×10^9	$<10^{-8}$	0.03	0.99	0.38
Toto:*ts*24B	<10	8.0×10^8	$<10^{-8}$	0.07	0.99	0.79
Toto:*ts*24B1	<10	1.5×10^9	$<10^{-8}$	0.12	1.18	0.89
Toto:*ts*24B3	2.4×10^9	2.7×10^9	8.2×10^{-1}	1.16	1.16	1.14
*ts*24R	7.0×10^8	2.6×10^9	2.7×10^{-1}	1.11	1.14	1.18
*ts*11	4.0×10^5	9.0×10^8	4.4×10^{-4}	0.03	0.73	0.47
Toto:*ts*11A1	4.4×10^5	1.5×10^9	2.9×10^{-4}	0.04	0.94	0.55
*ts*7	<10	2.0×10^9	$<10^{-8}$	0.02	1.16	0.53
Toto:*ts*7B	<10	5.8×10^9	$<10^{-8}$	0.02	1.12	0.70
Toto:*ts*7B1	8.2×10^6	1.0×10^9	8.2×10^{-3}	0.38	1.10	0.80
Toto:*ts*7B3	2.0×10^6	1.7×10^9	1.2×10^{-3}	0.05	1.16	0.71
*ts*7R	3.0×10^6	7.2×10^8	4.2×10^{-3}	0.50	1.25	1.20
*ts*18	4.0×10^5	1.6×10^9	1.6×10^{-4}	0.02	1.30	0.37
Toto:*ts*18B1	2.4×10^4	2.7×10^9	2.7×10^{-5}	0.05	1.52	0.56

[a] EOP, Efficiency of plating = titer (PFU/ml) at 40°C/titer (PFU/ml) at 30°C.
[b] Relative to that by Toto1101 (=1.00).

Characterization of rescued mutations. In order to establish that the mutations mapped are the ones responsible for the phenotypes previously described for these mutants and in order to characterize the phenotypes of these mutations in a uniform background, recombinant viruses containing a defined region from each of the *ts* mutants in a Toto1101 background were studied. Monolayers were transfected with RNA transcribed from the recombinant plasmids described earlier, and a single plaque of each virus was isolated from the 30°C plate and used to obtain a stock of the rescued mutant.

The virus titers of these stocks were determined at 30 and at 40°C, and the results are shown in Table 3. The virus stocks derived from the infectious transcripts of all but *ts*7 showed approximately the same apparent reversion frequency as did the parental viruses. (In *ts*21 and *ts*18, the apparent reversion frequency of the parental stock was somewhat higher than that of the stocks derived from the infectious transcripts because the parental stocks had not been as recently plaque purified; revertants are amplified in most stocks of *ts* mutants during passage because they have a selective advantage even at the permissive temperature.) For *ts*17, *ts*21, *ts*11, and *ts*18, this apparent reversion frequency was between 10^{-3} and 10^{-5}, consistent with the temperature sensitivity arising from a single-nucleotide change, which reverts with a frequency on the order of 10^{-4} (16, 28, 35). The results obtained with *ts*24, however, demonstrated a much lower reversion frequency. It is clear that the virus derived from the construct Toto:*ts*24B1 differs from Toto1101 by a single-nucleotide change (Fig. 2), and yet the reversion frequency was very low (less than 10^{-8}), as was the case for the parental *ts*24. Thus, it seems clear that in some cases a single-nucleotide change can revert to the parental nucleotide very infrequently, and, although in general the rate of nucleotide substitution in RNA virus genomes is quite high, that there are nucleotide substitutions which in certain contexts revert very infrequently. Another example of a specific mutation in the Sindbis virus genome with a very low reversion frequency has been described by Durbin and Stollar (9). The results shown in Table 3 also demonstrate that the virus derived from construct Toto:*ts*24B3 which contained the Val-to-Ala substitution in nsP3 was temperature insensitive with respect to plaquing efficiency and RNA synthesis at 40°C (see below).

Because of the very low reversion frequency of *ts*24 it was difficult to obtain a revertant, but one was obtained by passaging the virus stock at 40°C in liquid culture for three passages followed by plaque assay at 40°C. Because of the tendency of revertants to accumulate in the virus population once they arise, this method led to the isolation of a revertant which was temperature insensitive for plaquing efficiency and RNA synthesis.

The results with *ts*7 are consistent with its being a double mutant. The parental virus (*ts*7) and the virus derived from construct Toto:*ts*7B demonstrated a very low reversion frequency, less than 10^{-8}. Virus derived from the individual construct Toto:*ts*7B1 or Toto:*ts*7B3, which contained a single change in nsP2 or two changes in nsP3, respectively, showed a much higher apparent reversion frequency, on the order of 10^{-2} to 10^{-3}; this is consistent with the temperature sensitivity of these viruses being due to a single-nucleotide alteration (although this high apparent reversion frequency suggests that there may be some leakage). Note that both of these virus constructs were in fact temperature sensitive for plaque formation at 40°C. As shown in Table 3 and as will be described in more detail below, the change(s) in nsP3 (i.e., Toto:*ts*7B3) led to temperature sensitivity in RNA synthesis, that is, an RNA^- phenotype, whereas the change in nsP2 resulted in an $RNA^{\pm}$ phenotype ($RNA^{\pm}$ is defined in reference 29).

In order to separate the two changes in nsP3 of *ts*7, we took advantage of a *Sal*I site at nt 4845 that is located between these two changes. Construct Toto:*ts*7B4 containing the Lys-to-Glu change at position 218 gave rise to virus with wild-type properties, i.e., efficient plaquing and synthesis of wild-type levels of RNA at 40°C (data not shown). Construct Toto:*ts*7B5 containing the Phe-to-Ser change at residue 312 gave rise to *ts* virus that proved to be RNA^-, and thus this substitution alone in nsP3 was responsible for the *ts* phenotype.

A partial revertant of *ts*7 (*ts*7R) that plaqued with an efficiency of 4×10^{-3} at 40°C was isolated. The virus was RNA$^{\pm}$ and made RNA at wild-type levels after a shift to 40°C (Table 3; see also below). As described above, *ts*7R was a pseudorevertant, containing all three amino acid changes found in *ts*7. Because of the unusual nature of this result, the sequence data were confirmed by preparing a new library of *ts*7R cDNA clones for sequence analysis, and the same three amino acid substitutions were found in this library. The position of the suppressing change has not been mapped.

RNA synthesis by the rescued mutants. To examine whether the rescued mutants were as defective in RNA synthesis as the parental viruses, RNA synthesis was analyzed after infection at 40°C, at 30°C, or at 40°C after a shift from 30°C. Virus from clone Toto1101, the parental mutants, and the rescued mutants were compared. Total viral RNA synthesis was analyzed by a cytoplasmic dot hybridization method as described previously (13, 38) by using a ^{32}P-labeled probe consisting of minus-strand RNA transcribed from the structural region of the virus. The values determined relative to RNA synthesis by virus recovered from Toto1101 are shown in Table 3.

In *ts*17, *ts*21, *ts*11, and *ts*18, the virus recovered from the constructs was RNA$^-$, as was the parental virus. In the case of *ts*24, the virus recovered from Toto:*ts*24B1 was also RNA$^-$ but did show a slightly elevated level of RNA synthesis at 40°C relative to the *ts*24 parent, and there may be other as yet unmapped mutations in the *ts*24 genome that lead to a decreased level of RNA synthesis at 40°C (see also below).

In the case of virus derived from Toto:*ts*7B3, which contained the defect in nsP3, the virus was RNA$^-$, as was the parental *ts*7. Virus derived from Toto:*ts*7B1 containing the defect in nsP2 was RNA$^{\pm}$, showing substantial RNA synthesis at 40°C.

These different virus strains were also tested for RNA synthesis after establishing infection at 30°C and shifting them to 40°C. Under these conditions all of the mutants studied made substantial amounts of RNA following the shift, from about 20% as much as Toto1101 virus in the case of *ts*17 to equivalent amounts in the cases of some of the other constructs. In the cases of *ts*17, *ts*21, *ts*11, and *ts*18, the recovered virus demonstrated the same level of RNA synthesis after the shift as did the parental virus. In the case of *ts*24, however, virus derived from Toto:*ts*24B or Toto:*ts*24B1 exhibited virtually full RNA synthesis after the shift, whereas that derived from the parental virus was about half as much, suggesting once again that the parental *ts*24 contains one or more unmapped mutations that depress RNA synthesis. Note that RNA syntheses after the temperature shift with Toto:*ts*24B and Toto:*ts*24B1 were about equivalent, suggesting that the change in the nsP3 region had little effect upon RNA synthesis.

In the case of *ts*7, the viruses recovered from the various constructs all showed a substantial level of RNA synthesis after the shift.

Complementation analysis of the rescued mutants. We examined the ability of the viruses recovered from these constructs to complement representative *ts* mutants from the other complementation groups of RNA$^-$ mutants (Table 4). Complementation values obtained were defined as the yield after mixed infection at the nonpermissive temperature divided by the sum of the yields after infection with each mutant alone under the same conditions. The absolute magnitude of the complementation index thus depended upon the extent of leakage of the parental viruses, since alphavirus complementation is inefficient, never exceeding 1 to 10% of a wild-type yield (6, 7, 29). Virus from the three group A mutants demonstrated ready complementation with representatives of group B (*ts*11) and group F (*ts*6) but showed only poor or marginal complementation with group G mutant *ts*18. Virus derived from the group B mutant *ts*11 demonstrated ready complementation with members of groups A, F, and G. Finally, the two members of group G which we examined complemented with representatives of groups B and F but not with members of group A. Complementation by the separated changes in *ts*7, represented by Toto:*ts*7B1 and Toto:*ts*7B3, was difficult to demonstrate because of the high reversion frequency or high degree of leakage of these mutants, which led to a high background during the complementation assays, although complementation appeared to occur with group B (*ts*11) and F (*ts*6) mutants but not with the group A mutant *ts*24.

TABLE 4. Complementation between Sindbis virus RNA$^-$ *ts* mutants

Group	Constructs	Complementation index[a] of mutant (group):				
		*ts*24 (A)	*ts*11 (B)	*ts*6 (F)	*ts*18 (G)	Parental viruses
A						
	Toto:*ts*17B1	ND[b]	191	195	4	1
	Toto:*ts*21A2	ND	20	11	2	1
	Toto:*ts*24B1	<1	7	10	2	<1
B						
	Toto:*ts*11A1	105	1	120	118	1
G						
	Toto:*ts*7B	<1	19	35	ND	<1
	Toto:*ts*7B1	<1	3	2	ND	1
	Toto:*ts*7B3	<1	4	18	ND	1
	Toto:*ts*18B1	2	55	125	<1	<1

[a] Defined as the yield of virus from mixedly infected cells at 40°C divided by the sum of the yields from cells infected by each mutant alone.
[b] ND, Not determined.

From these complementation results and from the finding that mutants in groups A and G both arose from changes in nsP2, it appears that the previous complementation demonstrated between *ts*7 and *ts*18 (group G mutants) with representatives of group A (29) represents intragenic complementation and that the group G mutants should be considered a subgroup of group A. We had hoped that the mutation in nsP3 of *ts*7 might define another complementation group, but in part because of the high background in complementation assays with this mutant, we were unable to do so (complementation with group A was not seen).

DISCUSSION

The nature of the *ts* lesions. We have localized the mutations responsible for the temperature-sensitive phenotype of mutants belonging to complementation groups A, B, and G. The mutations found are shown in Fig. 3, in which sequences of four or five alphaviruses are compared in the regions affected. The mutations in *ts*11, *ts*21, *ts*18, and *ts*24, as well as the change in nsP3 of *ts*7, affected conserved amino acids. In the cases of *ts*11 and *ts*21, as well as that of *ts*24, these conserved amino acids were found in domains that exhibited a high degree of conservation, whereas the changes in *ts*18 and *ts*7, although they affected conserved amino acids, were

```
        nsP1          T:ts11                                    378
SIN     ICDQMTGIMATDISPDDAQKLLVGLNQRIVINGRTNRNTN
SF      --------L---VT-E--------------V----Q----
RR      --------L---VT-E--------------V----Q----
ONN     --------L--EVT-E--------------V----Q----

        nsP2                  Y:ts21                            326
SIN     FNMMQLKVHFNHPEKDICTKTFYKYISRRCTQPVTAIVST
SF      --------N---    N---E-CH-S------R--------
RR      --L-----N---    ----Q-L--S------L-I------
ONN     -----M--NY--    N---Q-YH-S------L-------S

        nsP2    L:ts18 T:ts17 N:ts7                             541
SIN     WSELFPQFADDKPHSAIYALDVICIKFFGMDLTSGLFSKQ
SF      --TIITA-KE-RAY-PVV--NE--T-YY-V--D-----AP
RR      -DT VLA-RE-RAY-PEV--NE--T-YY-V--D-----A-
ONN     --QIVQA-KE-RAY-PEV--NE--TRIY-V--D-----KP

        nsP2          S:ts24                                    763
SIN     LNPGGTLVVKSYGYADRNSEDVVTALARKFVRVSAARPDC
SF      -K---I- MRA-----KI--A--SS-S---SSARVL----
RR      -K---S-LIRA------V--M---------SAFRVL--A-
ONN     -K---S-LIRA------T--R-ISV-G---RSSR-LK-Q-
MID     -K---C-LMRA------T--M--N------ASIRVL--A-

        nsP3                          S:ts7                     325
SIN     TVCSSTPLPKHKIKNVQKVQCTKVVLFNPHTPAFVPARKY
SF      V----F----YHVDG----K-E--L--D-TV-SV-SP---
RR      I----F----YR-EG----K-DR-LI-DQTV-SL-SP---
ONN     I----F----Y--EG----K-S-AL--DHNV-SR-SP-T-
MID     -----F----Y--PG--R-A-SA-M---HDV--L-SP---
```

FIG. 3. Comparison of amino acid sequences from four or five alphaviruses in the regions near *ts* mutations. Changes in amino acid sequences are indicated (↓) for each mutant. Sequence data are from the following sources: SIN (Sindbis virus), Strauss et al. (32); SF (Semliki Forest virus), Takkinen (37); RR (Ross River virus), Faragher et al. (10) and Strauss et al. (30); ONN (O'Nyong-nyong virus), Strauss et al. (30) and unpublished data; MID (Middelburg virus), Strauss et al. (31) and unpublished data.

found within domains that are not otherwise highly conserved. The change in nsP2 of *ts*7 is intriguing. This Asp→Asn substitution in nsP2 changed the Sindbis virus amino acid to that found in the other alphaviruses sequenced to date, and this change of the Sindbis virus amino acid to the consensus amino acid resulted in temperature sensitivity.

It is noteworthy that *ts*18, *ts*17, and *ts*7 affect amino acids in nsP2 over a region of only 14 amino acids. This region is not particularly well conserved among alphaviruses, but its precise sequence appears to be important because of the fact that multiple changes in this region lead to temperature sensitivity. In the course of this study we also found two changes in nsP3 which affected consecutive amino acids and which had no apparent phenotype, Lys-218→Glu in *ts*7 and Val-219→Ala in *ts*24. Neither of these amino acids is conserved in other alphaviruses, and the surrounding domain is not well conserved, suggesting that this region of nsP3 can accommodate a number of amino acid substitutions.

TABLE 5. Summary of mutations in Sindbis virus RNA⁻ *ts* mutants

Group and mutant	Mutated protein	Mutation[a]
A		
*ts*17[b]	nsP2	Ala-517 → Thr
*ts*21	nsP2	Cys-304 → Tyr
*ts*24[b]	nsP2	Gly-736 → Ser
B		
*ts*11	nsP1	Ala-348 → Thr
G		
*ts*7	nsP2	Asp-522 → Asn
	nsP3[c]	Phe-312 → Ser
*ts*18[b]	nsP2	Phe-509 → Leu
F[d]		
*ts*6	nsP4	Gly-153 → Glu
*ts*110	nsP4	Gly-324 → Glu
*ts*118	nsP2	Val-425 → Ala
	nsP4	Gln-93 → Arg

[a] Amino acids are numbered from the N terminus of each protein.
[b] Defect in cleavage of nonstructural proteins at nonpermissive temperature.
[c] Changes in nsP3 that do not result in temperature sensitivity (Val-219→Ala in *ts*24 and Lys-218→Glu in *ts*7) are not shown. (Note that the nsP3 change shown for *ts*7 [Phe-312→Ser] results in an RNA⁻ phenotype, whereas the nsP2 change [Asp-522→Asn], results in an RNA⁺ phenotype.)
[d] Group F changes are from Hahn et al. (13).

Mutants *ts*7, *ts*11, *ts*17, *ts*21, and *ts*24 were produced by mutagenesis with nitrosoguanidine (5). Of the six mutations in these five mutants that resulted in temperature sensitivity, five were G→A transitions and one was an A→G transition. Transition of G→A is the most common change to be expected from the action of nitrosoguanidine (8), although our past results on mapping of *ts* lesions in Sindbis virus have more often found other changes produced (2, 12, 13, 18). Mutant *ts*18 was produced by mutagenesis by nitrous acid. The U→C transition responsible for temperature sensitivity is not one of the changes expected to arise from HNO_2 treatment (8), but once again our previous mapping studies have often found other changes produced, including U→C transitions (2, 12, 13).

From our mapping studies, the gene order for the complementation groups is NH_2-B-(A/G)-(G)-F-COOH. These results differ from the results of Fuller and Marcus (11), in which the complementation groups were ordered NH_2-G-A-(B/F)-COOH, as determined from the relative rate of UV inactivation of the ability of the mutants to complement. The major discrepancy between the two orders is the location of complementation group B, and it is unclear why the UV inactivation data placed this complementation group in the wrong position.

Functions of the nonstructural proteins. All four RNA⁻ complementation groups have now been assigned to nonstructural proteins, and these results are summarized in Table 5. From these data hypotheses can be developed for the functions of the nonstructural proteins during RNA replication. First, it is clear that mutations in any of the four nonstructural proteins can lead to an RNA⁻ phenotype, implying that all four proteins are important for RNA replication (note that the change in nsP3 of *ts*7 is responsible for

the RNA⁻ phenotype of this mutant). Second, at least some of the functions of nsP1, nsP2, and nsP4 can be deduced from the phenotypes of the RNA⁻ mutants that have been characterized. The results of studies of the group F mutants *ts*6 and *ts*110 are consistent with the hypothesis that nsP4 is the polymerase or major elongation component of the viral replicase (13). Previous studies of the single group B mutant, *ts*11, had shown that minus-strand RNA synthesis ceased when infected cultures were shifted to 40 from 30°C, whereas synthesis of both 49S and 26S plus-strand RNA continued unabated (24). This suggests that nsP1 may function as an initiation factor for synthesis of minus-strand RNA.

The possible functions of nsP2 deduced from previous studies of the group A and G mutants are more varied, and it is probably the case that each of the virus nonstructural proteins has more than a single function during RNA replication. *ts*17, *ts*21, and *ts*24 of group A (as well as *ts*133, which has not yet been mapped) and *ts*18 of group G have been found to be temperature sensitive in the synthesis of 26S RNA upon a shift from a permissive to a nonpermissive condition (17, 23), suggesting that nsP2 may be required for the initiation of 26S RNA synthesis. In addition, three of the mutants with changes in nsP2, *ts*17 and *ts*24 of group A and *ts*18 of group G (Table 5) fail to process the polypeptide precursor P123 (also known as ns230) upon shift to the nonpermissive temperature (17, 23; W. R. Hardy and Y. S. Hahn, unpublished data). This suggests that nsP2 is the protease that processes the nonstructural polyprotein precursors. Two of the mutations in nsP2 that resulted in failure to cleave the polyprotein precursor, those in *ts*17 and in *ts*18, were found close to one another (Fig. 3), suggesting that this region might form part of the protease, although the mutation in *ts*7 was also found within the same region and did not affect proteolytic processing. The mutation in *ts*24 which also led to failure of processing was found some 200 amino acids downstream, suggesting that this region might also form part of the protease and that nsP2 is folded so as to bring these regions together to form an active site. We presume that the enzyme is a cysteine protease and propose that one of the conserved cysteines in the domains defined by the *ts* lesions is the active residue. It is also possible, however, that these various mutations affect protease function because they alter the overall folding of the protein rather than because they are situated near the active site.

*ts*17, *ts*133, and *ts*24 of group A were also found to have a third phenotypic defect, namely, that upon a shift from a permissive to a nonpermissive temperature the normal shutdown of minus-strand synthesis failed to occur (23, 25, 26). In these mutants, not only did this shutdown fail to occur at a nonpermissive temperature, but shifting infected cells from permissive to nonpermissive conditions after shutdown of minus-strand synthesis resulted in resumption of minus-strand synthesis. ts^+ revertants of *ts*17 and *ts*133 demonstrated normal shutoff, whereas ts^+ revertants of *ts*24 did not. Thus, in the case of *ts*24 the defect in shutdown appears to arise from an unmapped mutation, consistent with the results presented in Table 3 implying that *ts*24 has unmapped defects related to RNA synthesis. Further studies on these mutants and their revertants will be required to ascertain whether the regulation of minus-strand synthesis is a function of nsP2; the results obtained with *ts*17 suggest that it is, although another protein (defined by the unmapped change in *ts*24) may be involved as well.

Thus it appears that alterations in nsP2 can result in temperature-sensitive synthesis of 26S RNA, temperature-sensitive proteolysis of nonstructural proteins, and temperature-sensitive regulation of minus-strand synthesis, implying that this protein is involved in all of these functions. However, the temperature-sensitive synthesis of 26S RNA does not in itself necessarily lead to the temperature-sensitive phenotype, defined as a failure to form plaques at the nonpermissive temperature, since a ts^+ revertant of *ts*133 examined remained temperature sensitive in 26S RNA synthesis (25), and as discussed above for *ts*24, the temperature-sensitive regulation of minus-strand synthesis does not necessarily result in temperature sensitivity of plaque formation. All of the ts^+ revertants of proteolysis mutants examined, however, did show normal processing, so that in the case of these mutants the temperature sensitivity might result from the failure to process the nonstructural proteins at elevated temperatures. From the results obtained with *ts*24 and *ts*133, and because other RNA⁻ mutants in group A show none of these phenotypes, it appears clear that nsP2 must possess other functions that are also required for RNA replication.

It has been shown that nonstructural proteins nsP1, nsP2, and nsP4 of Sindbis virus share amino acid sequence homology with nonstructural proteins from several RNA plant viruses, including alfalfa mosaic virus, bromegrass mosaic virus, and tobacco mosaic virus (1, 15). The plant viruses share similarities in replication strategies with the alphaviruses, including production of a subgenomic messenger RNA for the translation of structural proteins as well as the obvious need to produce genomic-length plus and minus strands. The amino acid sequence similarities suggest that the plant virus proteins perform the same functions during replication of the virus RNAs as do the corresponding proteins of Sindbis virus, and it will be of interest to compare the functions of these proteins as more information becomes known.

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